

**ULTRASENSITIVE DETECTION OF PATHOGENIC MICROBES**

**Cross-Reference to Related Applications**

[0001] This application is based on and claims priority to U.S. Provisional Application No. 60/428,914, filed 11/26/2002, fully incorporated herein by reference.

**Field of the Invention**

[0002] The present invention relates to improved methods and reagents for detecting the presence of pathogenic microbes in water and clinical samples.

**Background of the Invention**

[0003] As human population density increases as a result of urban growth, and animal population densities increase from intensive agri-business practices, the pressures on water resources can rise dramatically. Pollution in the form of sewage from human populations, or from livestock in agricultural operations, can lead to elevated levels of microbial contamination in drinking water, irrigation water and ground water, resulting in pathogen contamination of food and recreational water resources. The coliforms including *E. coli* cause a variety of ailments in humans and domesticated animals, most noticeably urinary tract infections, gastroenteritis, and selected skin disorders.

[0004] Traditionally coliforms have been detected and quantified by enzymatic and culturing methods such as the multiple-tube fermentation (MTF) technique to yield most probable number (MPN) or by membrane filtration (MF) and culturing techniques (APHA, 1995; Rompré et al., 2002). Among the drawbacks of these traditional methods is the detection of false positives and the need for further confirmative tests and the long time (on the order of days) and labour required to conduct these tests (Rompré et al., 2002). With culture-based techniques there is also the potential risk of not detecting cells that are metabolically active, but not culturable

(viable but not culturable; VBNC). PCR is an efficient method for detection of VBNC cells (Tamanai-Shacoori et al., 1996). PCR-based detection methods can therefore overcome false negatives obtained with culture-based detection methods, and can overcome false positives from some tests due to the sequence-based specificity of PCR testing.

[0005] Endpoint PCR has been established as a qualitative method to measure the presence or absence of any given pathogen, including coliforms and has been applied to this problem in the early 1990s (Bej et al. 1990a, 1990b, 1991a, 1991b). A number of gene probes were successful in the studies conducted by Bej et al., including *lacZ* (total coliforms), *uidA* (*E. coli*), and *lamB* (*E. coli*, *Salmonella*, *Shigella*), and results indicated that the PCR methodologies were as good as, or even more reliable than plate counts or defined substrate methods (Bej et al. 1990a, 1990b). These approaches are reliable, but they are still more time consuming and qualitative in nature than the quantitative measurements that can be obtained with the application of 5' nuclease PCR to the science of microbial water quality testing.

[0006] *E. coli* O157:H7, EHEC (enterohaemorrhagic *E. coli*) is an important water- and foodborne pathogen that can cause a variety of human diseases (**Karmali, 1989**; **Willshaw et al., 1994**). It is differentiated from resident microflora by specific biochemical characteristics, such as the inability to ferment sorbitol in 24 hr (Farmer et al., 1985) and the lack of  $\beta$ -glucuronidase activity (Doyle and Schoeni, 1984). Injured or stressed bacteria may not grow on selective media or may not express the antigen required for immunological detection. Immunological methods rely on the specific binding of an antibody to an antigen, for example the interaction of antigens such as lipopolysaccharide (LPS) or Shiga-like toxins (SLTs) with specific antibodies. Conventional and immunological methods are sensitive and permit low numbers of bacteria ( $\sim 10^3$  cells ml<sup>-1</sup>) to be detected in complex sample matrices. However, the immunological methods do not distinguish between live or dead cells and conventional cultural and immunological methods are often not appropriate for detection of injured or stressed bacteria. *E. coli* O157:H7 is often present at very low levels, masked by a high population of resident microflora, making the pathogen difficult to detect and subsequently distinguish phenotypically.

[0007] There are numerous virulence markers in EHEC (enterohaemorragic *E. coli*), they include SLTs (Acheson, 2000), intimin, hemolysin, and the locus of enterocyte effacement (Feng et al., 2001a). Food-borne illnesses have occurred with isolates that possess all or only a few of these markers (Feng et al., 2001b). EHEC strains containing *slt1* and *slt2* have been isolated from patients with hemorrhagic colitis, studies have shown that strains possessing only *slt2* are more frequently associated with human disease complications (Restino et al., 1996). *E. coli* possessing *slts* are often referred to as Shiga toxin-producing *E. coli* (STEC). The *eaeA* gene has been shown to be necessary for the production of attaching and effacing lesions that are a characteristic of enteropathogenic *E. coli* (EPEC) (Jerse et al., 1990). The *slt1*, *slt2* and the *eae* genes have been cloned and sequenced (Jackson et al., 1987; Yu and Kaper, 1992) and the characterization of these virulence factors has led to a better understanding of the pathogenesis of diarrheal diseases caused by these organisms, providing a new dimension to their identification. The *slt* genes and the *eaeA* gene have been used for detection with genetic probes and by PCR (Frantamico et al., 1995; Deng and Frantamico, 1996; Germani et al., 1997; Meng et al., 1997). Other genes used for the identification of *E. coli* O157:H7 by PCR assays include *stx1*, *stx2* (Gannon et al., 1992), *eae* (Schmidt et al., 1994), *rfbE* (Desmarchelier et al., 1998) and *fliC* (Fields et al., 1997; Gannon et al., 1997). Endpoint PCR amplification of *eaeA* was first reported as a diagnostic tool for the detection of toxigenic *E. coli* O157:H7 by (Gannon et al., 1993). *EaeA* encodes intimin, a 97 kDa outer membrane protein (Louie et al., 1993). The 5' end of the *eaeA* gene (first 2200 bases) is 97% homologous among EPEC, whereas the last 800 bp of the 3' end are variable among the strains (Beebakhee et al., 1992; Louie et al., 1994). Applied Biosystems Inc. (ABI) has designed a 5' nuclease PCR-based diagnostic kit for detection of pathogenic *E. coli* O157:H7 that will produce plus/minus results with respect to contamination (ABI, 2000). The gene target for this kit is a region of unknown function upstream of the *eaeA* gene. 5' nuclease PCR and multiplex endpoint PCR have been used for the detection of *E. coli* O157:H7 in meat with various regions of the *eaeA* gene (Oberst et al., 1998; Call et al., 2001). The 3' end of the *eaeA* gene was targeted for the detection of *E. coli* O157:H7 in beef using endpoint PCR (Sharma et al., 1999; Uyttendaele et al., 1999). Many PCR-based detection techniques use the *stx1* and *stx2* genes, for detecting *E. coli* O157:H7 (Jothikumar and Griffiths, 2002). However, not

all strains of this pathogen have both or either of these genes (Karch et al., 1996; Kim et al., 1998; Feng et al., 2001a). Moreover exploiting multiplex PCR protocols to amplify different genes encoding the virulence factors, with different specific primers, could be a good predictor of the pathogenic potential of *E. coli* strains.

[0008] Polymerase chain reaction-based assays are specific, can be extremely sensitive and results are obtained in a few hours. However, they detect chromosomal gene sequences which can be present in viable and dead cells and, therefore, no determination can be made concerning the presence of only viable cells in a sample (Josephson et al., 1993; Masters et al., 1994). This is a decided disadvantage of PCR-based methods. Several options are available to eliminate the risk of detecting nucleic acid from non-viable cells by PCR, such as reverse-transcription of sample isolated RNA (RNA is less stable than DNA and would be indicative of viable cells in the sample). Several types of RNA are produced in bacterial cells, including ribosomal RNA (rRNA) and messenger RNA (mRNA). rRNA is a universal constituent of bacterial ribosomes and is present in high copy numbers but, similar to DNA, rRNA can persist for an extended period in dead cells (Uyttendaele et al., 1997; McKillip et al., 1998). Messenger RNA is considered a more appropriate target as an indicator of viability since most mRNA species have a short half-life of only a few minutes (Kushner, 1996).

[0009] A recent study (Yaron and Mathews, 2002) examined the expression of seven genes of *E.coli* O157:H7 (*rfbE*, *fliC*, *stx1*, *stx2*, *mobA*, *eaeA* and *hly*) under a range of conditions to determine a suitable mRNA target(s) for reverse transcriptase (RT)-PCR amplification. Detection based on PCR amplification of these genes has been reported previously (Schmidt et al., 1994; Fields et al., 1997; Desmarchelier et al., 1998). The expression of genes and stability of mRNA were evaluated for samples collected under typical growth conditions, prior to and after thermal treatment of 121°C for 15 min and 60°C for 20min and in cells from a sample (suspension of bacteria in water) which decreased to an undetectable level (<0.1 cfu ml<sup>-1</sup>) as determined by plate count but contained viable cells based on cytological analysis. The results of RT-PCR amplification indicate that, in most cases, the *rfbE* gene can be used for detection of viable *E. coli* O157:H7.

[0010] Microcystin-producing cyanobacteria are also a serious threat to both animal and human health due to the toxicity of non-ribosomally produced proteins. This toxin is encoded by the polycistronic microcystin synthetase operon (Nishizawa et al., 1999, 2000). Microcystin phycotoxins, are one of the most common natural biotoxins in fresh as well as marine waters (Andersen et al., 1993; Codd, 1994, 1995; Bury et al., 1997; Sivonen and Jones, 1999). Microcystin is a cyclic heptapeptide produced by toxic strains of *M. aeruginosa*, as well as species of *Anabaena*, *Nostoc*, and *Oscillatoria* (Codd, 1995; Sivonen and Jones, 1999). This peptide is hepatotoxic and acts by inhibiting protein phosphatases type 1 and 2A, which are tumor suppressors (Sivonen and Jones, 1999), and it has been directly associated with the production of liver cancer in humans, fish, and livestock. Microcystin toxin levels are increasing in the Great Lakes as a result of a number of factors including selective filtration by zebra mussels (Vanderploeg et al., 2001).

[0011] There are a number of different methodologies currently in use to detect the toxin. These include high-performance liquid chromatography (HPLC), mass spectrometry, ELISAs (Chu et al., 1989), and other enzyme-based methods, which can be applied to water, cyanobacterial scums and clinical material (Codd et al., 1994). ELISAs offer a relatively narrow range in which microcystin can be quantitated in samples. Relative to ELISAs, HPLC is a relatively time-consuming process. Neither of these assays can distinguish between the toxic and non-toxic variant of microcystis. None of the above methods are capable of detecting the presence of the pathogen itself, as we are able to with real-time PCR. The ability to detect the toxin-producing pathogen itself, rather than the toxin would allow pro-active control of microcystin-producing cyanobacteria in water. Competitive endpoint PCR has been used for the quantification of *Microcystis* in water by amplification of the 16SrDNA sequence, and subsequently didioxy fluorescein cycle labeled, followed by chromogenic detection (Rudi et al., 1998).

[0012] *G. lamblia* (known also as *G. intestinalis* and *G. duodenalis*) and *C. parvum* are protozoan parasites that cause severe diarrheal illness in human hosts. Symptoms include profuse watery diarrhea, nausea, cramps, malabsorption and last for 2 or more weeks (Vesy and Peterson, 1999; Chen et al., 2002). While infections are usually self-limiting in immunocompetant individuals, chronic infections can be life-threatening in

immunocompromised individuals, such as AIDS patients. Metronidazole is the standard treatment against *Giardia* infection, however, no suitable antimicrobial agent exists to eradicate *Cryptosporidium*.

[0013] Ninety percent of transmission of these pathogenic protozoans is through water while 10% occurs through food (Rose and Slifko, 1999). The incidence of foodborne outbreaks due to protozoan pathogens is likely underestimated due to the difficulty in detection of low numbers of organisms, as enrichment techniques cannot be used. Detection of *Giardia* and *Cryptosporidium* on domestic, fresh vegetables and fruits in Norway (Robertson and Gjerde, 2001), a wealthy and modern country, have important implications for food safety in North America.

[0014] Infection with these protozoans is initiated through the ingestion of the cyst stage of *Giardia* or oocyst stage of *Cryptosporidium*. These transmission stages are very hardy and can persist in the environment for a month (*Giardia*) or several months (*Cryptosporidium*). While their abundance in water is very low, from 0.5-200/100L water with an average of 25 cysts/100L (Wallis et al., 1994; Payment et al., 2000; Thurston-Enriques et al., 2002), the infective dose is also very low (10 cysts/oocysts; Rendtorf, 1954; DuPont et al., 1995). Thus, very sensitive techniques are required to detect cysts/oocysts in the environment. There are no standard collection methods for concentration of *Giardia* or *Cryptosporidium* from environmental samples, however, the USA EPA recommends the use of method 1623 involving filtration through Envirocheck filters and immunomagnetic bead separation (USA EPA, 1999). This procedure is very costly (>\$100/sample) and filtration of water samples through envirocheck filters (Pall Gelman) is not very efficient, ranging from 15% (Simmons et al., 2001). Other methods, filtration through 3µm cellulose nitrate and 1.2 µm cellulose acetate (Sheppard and Wyn-Jones, 1996) are much less expensive (\$1/filter) and are as efficient as the Envirocheck. An alternative method has been described for simultaneous collection of protozoa, bacteria and viruses using ultra filtration membranes. The microza ultra filtration system has efficiencies of recovery of *Cryptosporidium* of 30-80% from environmental water samples (Kuhn and Oshima, 2001). These filters are reusable and come in different sizes to accommodate 2-1000L volumes of water (Pall Gellman).

[0015] Cysts and oocysts are resistant to many environmental stresses and to disinfection, such as chlorination, used in water treatment practices. Distinguishing live from dead cells is important in determining water treatment effectiveness and risks to public health. Current methods for viability determination include animal infectivity (Black et al., 1996; Neumann et al., 2000), vital dye staining (Belosevic et al., 1997), excystation (Rose et al., 1988) and *in vitro* cultivation combined with PCR (Rochelle et. al., 1997; Rochelle et al., 2002; Di Giovanni et al., 1999). Reverse transcription PCR (RT-PCR) enables measurement of mRNA to detect viable cells and has been used to determine *G. lamblia* and *C. parvum* viability (Mahbubani et al., 1991; Stinear et. al., 1996; Jenkins et al., 2000).

[0016] Domestic animals, pets and wildlife act as reservoirs of *Giardia* and *Cryptosporidium* (Thompson, 2000; Heitman et al., 2002; Dillingham et al., 2002). A comparative study of sources of *Giardia* and *Cryptosporidium* from humans (sewage influent), agriculture (farms) and wildlife (scats) found that the lowest prevalence was in wildlife and the highest in human sewage. However, the highest concentrations of these protozoans were from calf-cow sources (Heitman et al., 2002). Prevalences of *Giardia* and *Cryptosporidium* on farms range from 9-40% in cattle, sheep, pigs and horses (Olsen et al., 1997). There is considerable genetic diversity within *G. lamblia* and *C. parvum* and both can be subdivided into major genotypes, each containing sub-genotypes. The major genotypes of *G. lamblia* are assemblages A and B; A is associated with a mixture of human and animal isolates and B is predominately associated with human isolates (Thompson et al., 2000). The greatest potential for zoonotic transmission of *Giardia* is with assemblage A genotypes. A similar pattern exists with *C. parvum* isolates, whereby genotype 1 contains predominately human isolates and genotype 2 contains bovine isolates (Dillingham et al., 2002). Knowledge of genotype can assist in identification of source of waterborne outbreaks for predictive epidemiology.

[0017] Methodologies for identifying pathogenic *Giardia* and *Cryptosporidium* are not nearly as well defined as for bacterial identification. They rely primarily on microscopic identification of intact cysts, requiring an expert in identification, time for staining the cells, preparing slides and examination. Stains for detection of cells include dyes such as Lugol's stain and immunofluorescent stains (e.g. Dynabeads G-C

combo kit form Dynal Ltd. and Aqua-Glo G/C Direct, Waterborne Inc.). Other methods for detection of intact cysts or oocysts involve using fluorescent antibody labeling and detection by flow cytometry. Enzyme immunoassay kits are available on the market and take 2-3 hr to perform (Prospect T/Cryptos, Alexon Inc. and *Giardia* Celisa, CELLABS PTY LTD). Recently, a rapid antigen based kit (ColorPAC™, BD) for detection of *Giardia* and *Cryptosporidium* was recalled by the manufacturer due to false positives (MMWR, 2002). None of these techniques provide the ability to genotype.

[0018] PCR has been used to detect *Giardia* and *Cryptosporidium* in waste, ground and treated waters (Johnson et al., 1995; Stinear et al., 1996; Kaucner and Stinear, 1998; Chung et al., 1998), sewage sludge (Rimhanen-Finne et al., 2001), soil (Walker et al., 1998; Mahbubani et al., 1998), food (Laberge et al., 1996) and stool (Morgan et al., 1998; Webster et al., 1996; Gobet et al., 1997). PCR is equally or more sensitive than immunofluorescent antibody (IFA) in detection of these pathogens (Mayer and Palmer, 1996; Morgan et al., 1998) and has the capability for high throughput processing of samples resulting in significant reduction in costs.

[0019] Real-time PCR detection of *Cryptosporidium* has recently been reported. The primer/probe sequences have been based on: the Cp11 rRNA and 18s rRNA genes (Higgins et al., 2001); an unidentified gene segment generated by the random amplified polymorphic DNA (RAPD) technique (MacDonald et al., 2002); an oocyst wall protein encoding gene (Fontaine and Guillot, 2002); a highly polymorphic region of the SSU rRNA (Limor et al., 2002) and β-tubulin (Tanriverdi et. al., 2002). To date there have been no reports of the use of real-time PCR for detection of *Giardia*.

[0020] Traditional methods of bacterial detection in foods rely on cultivation of bacteria from the food matrix. While these procedures are very sensitive they can take days to produce results. Enzymatic and molecular approaches are much more rapid but the sensitivity of detection,  $10^3$  to  $10^4$  CFU/gm, is typically less than cultivation (Jaykus, 2003). Rapid techniques for concentrating and isolating bacteria from food matrixes (carcass swabs) and rapid detection of the bacteria using real-time PCR (qPCR) would greatly benefit the public by increasing the safety of their food.

[0021] From the preceding, it will be appreciated that there is an acute need for methods and reagents that enable the rapid and accurate detection of pathogenic microbes not only in environmental samples but, failing their detection and reduction, also in clinical samples of infected individuals to enable proper and rapid medical treatment. This need is especially acute with respect to total coliforms (as a water quality indicator) and such pathogenic microbes as *E. coli* O157:H7, the microcystin-producing cyanobacteria including *M. aeruginosa*, and the protozoan parasites including *Cryptosporidium* such as *C. parvum* and *Giardia* including *G. lamblia*. It is accordingly an object of the present invention to provide methods and reagents useful in their detection.

#### Summary of the Invention

[0022] In one aspect, the present invention provides a method useful to detect a pathogenic microbe, the method comprising the step of subjecting a DNA sample that is either extracted from said microbe or is a cDNA equivalent to a polymerase chain reaction comprising primers adapted to produce and amplify a detectable amplicon from a gene responsible for the pathogenicity of said microbe, and measuring in real time the accumulation of said amplicon during said reaction. In a preferred embodiment of the invention, to render the amplicon detectable during the reaction, the polymerase chain reaction is performed in the presence of both an enzyme having 5' nuclease activity (a 5' nuclease) and a probe having a detectable label released following cleavage of the probe by the action of the 5' nuclease.

[0023] In another aspect, the present invention provides a multiplexed method useful to detect at least two different pathogenic microbes in a given sample, the method comprising the step of subjecting a sample comprising DNA extracted from said microbes, or a cDNA equivalent thereof, to a polymerase chain reaction comprising primers adapted to produce and amplify detectable amplicons that are different for each pathogenic microbe, and measuring in real time the accumulation of said amplicons during the reaction. Desirably, the multiplexed method also utilizes the 5' nuclease susceptible probes to detect and measure accumulation of the amplicons.

[0024] For the detection of specific pathogenic microbes, the present invention further provides oligonucleotide primers and oligonucleotide probes useful in a polymerase chain reaction to detect the presence of a selected pathogenic microbe.

[0025] In embodiments of the present invention, there is provided an amplicon having a nucleotide sequence selected from *the coding region of*:

- (a) the region spanning residues 2574-2895 of the *lacZ* gene of *E. coli*;
- (b) the region spanning residues 2673-2759 of the *eaeA* gene of *E. coli* O157:H7;
- (c) the region spanning residues 1438-1559 of the *mcyA* gene of *Microcystis aeruginosa*;
- (d) the region spanning residues 222-296 of the  $\beta$ -giardin gene of *G. lamblia*;
- (e) the region spanning residues 411-485 of the  $\beta$ -giardin gene of *G. lamblia*; and
- (f) the region spanning residues 583-733 of the COWP gene of *C. parvum*.

[0026] In other embodiments of the present invention, the primers and probe are adapted to detect total coliforms (tested with *E. coli*). In a specific embodiment, the primers are designed to produce an amplicon from the *E. coli lacZ* gene, which preferably is a 142bp amplicon spanning residues 2574 and 2895 (numbered with reference to GenBank Accession: V00296). In other embodiments of the invention, there are provided primers useful in the amplification of that amplicon of the *E. coli lacZ* gene, which are selected from the primers identified in Table 2 herein as SEQ ID NOs: 4 and 5. In another embodiment, the present invention provides a probe useful to detect the amplicon resulting from said primers, the probe having SEQ ID NO.6. In a preferred embodiment, the probe incorporates one or more labels that are released for detection when the probe is cleaved by an enzyme having 5' nuclease activity. With these reagents, the present method can be applied for the detection of coliforms, including *E. coli* strains capable of causing intestinal disease.

[0027] In another embodiment of the invention the primers and probe are adapted to detect *E. coli* O157:H7. In a specific embodiment, the primers are designed to produce an amplicon from the *eaeA* gene, which preferably is an 87 bp amplicon located between residues 2673 and 2759 (numbered with reference to GenBank Accession: X60439). In other embodiments of the invention, there are provided primers useful in producing an amplicon of the *eaeA* gene, which are selected from the primers identified in Table 2 herein as SEQ ID NOs: 1 and 2. In another embodiment, the present invention provides a probe useful to detect the amplicon resulting from said primers, the probe having SEQ ID NO.3. In a preferred embodiment, the probe incorporates one or more labels released for detection when the probe is cleaved by an enzyme having 5' nuclease activity.

[0028] In another embodiment of the invention the primers and probe are adapted to detect microcystin-producing cyanobacteria, and particularly *M. aeruginosa*. In a specific embodiment, the primers are designed to produce an amplicon from the *mcyA* gene from the microcystin synthetase gene operon, which preferably is a 122 bp amplicon spanning residues 1438 and 1559 (numbered with reference to Gen Bank Accession: AB019578). In other embodiments of the invention, there are provided primers useful in producing an amplicon of the *mcyA* gene, which are selected from the primers identified in Table 2 herein as SEQ ID NOs: 7 and 8. In another embodiment, the present invention provides a probe useful to detect the amplicon resulting from said primers, the probe having SEQ ID NO.9. In a preferred embodiment, the probe incorporates one or more labels that are released for detection when the probe is cleaved by the action of an enzyme having 5' nuclease activity.

[0029] In still another embodiment of the invention, the primers and probes are adapted to detect pathogenic protozoans including *Giardia* and particularly *G. lamblia*, as well as *Cryptosporidium* including *C. parvum*. With respect to detection of *G. lamblia*, the primers are designed to produce an amplicon from the β-giardin gene. One set of primers, herein referred to infra as the P241 set, yields a 74bp amplicon spanning residues 222-296 (CDS of GenBank Accession # M36728). In specific embodiments, the primers are selected from the primers identified in Table 2 herein as SEQ ID NOs: 10 and 11. In another embodiment, the present invention

provides a probe useful to detect the amplicon resulting from said primers, the probe having SEQ ID NO.12. In other embodiments, the primers are designed to produce a 74bp amplicon spanning residues 411-485 (CDS of GenBank Accession #M36728) of the  $\beta$ -giardin gene, and the primers, designated P434 herein, are selected from the primers identified in Table 2 by SEQ ID NOs. 13 and 14. A suitable probe for such an amplicon has the sequence represented by SEQ ID NO. 15, in Table 2 *infra*.

[0030] For detection particularly of *C. parvum*, the primers are designed to produce an amplicon from the *Cryptosporidium* oocyst wall protein, designated COWP. The primers suitably are designed to produce a 151bp amplicon spanning residues 583-733 (CDS of Gen Bank Acc#Z22537). In specific embodiments, the primers are selected from the primers identified in Table 2 herein as SEQ ID NOs: 16 and 17. In another embodiment, the present invention provides a probe useful to detect the amplicon resulting from said primers, the probe having SEQ ID NO.18.

[0031] It will be appreciated that the present invention also embraces amplicon-binding sequence variants of the primers and probes herein described. Such variants may include substitution of from 1-5 nucleotides in the noted sequences. The substitutions are selected to minimize loss in binding affinity for the amplicon that results from the substitution, relative to the actual sequences herein provided.

[0032] It will also be appreciated that the primer and probe sets herein described will be useful to produce amplicons having some variation, say up to 20% variation, from the specific amplicon sequences herein described. While some specificity may be sacrificed, the method nevertheless will still detect pathogen strains having minor variation in the sequence targeted for amplification and detection.

[0033] It is to be appreciated that while the method of the present invention preferably utilizes a real time, 5' nuclease-based polymerase chain reaction to produce and detect the amplicon targeted within the microbial genome, the primers and probes herein described can also be used in polymerase chain reactions and related procedures that utilize different strategies, including RT-PCR, end-point PCR, NASBA and the like. In this vein, it will further be appreciated that the substrate DNA can either be extracted from the microbe(s) present in the sample, or it can be synthesized from

extracted RNA using standard methods of cDNA preparation. Alternatively, the extracted RNA can serve as the intermediary of an otherwise DNA-based amplification method. In the NASBA approach, for instance, the given amplicon can be produced using the reverse primers herein described, but using a forward primer adapted by addition 5' of 25bp constituting the sequence for T7 promoter. In this approach, the same probe sequence can also be employed, but incorporating a molecular beacon probe instead of the Taqman probe.

[0034] It will thus be appreciated that the present invention is particularly adapted for the rapid, sensitive and selective detection, in real time, of a variety of pathogenic microbes in both environmental and clinical specimens. Embodiments of the present invention are particularly adapted for the detection of total coliforms, *E. coli* O157:H7, toxicogenic *M. aeruginosa*, *G. lamblia*, and *C. parvum*.

[0035] In addition, the present invention provides improvements in procedures by which DNA samples are collected, in methodology for managing inhibitory substances in the samples, and in methods for discriminating between live and dead cells within a sample. These improvements permit analysis of a wider array of microbial samples, including finished drinking water, sewage, waste water, treated water, disinfected water, irrigation water, and water obtained from wells, rivers, lakes and recreational waters such as swimming pools. Other samples that can be analyzed by the present method include food (such as fruits, vegetables, meat and prepared food items), swabs taken from slaughter lines, and meat surfaces, as well as swabs taken from environmental surfaces from slaughter houses, and meat preparation facilities, soil and clinical and veterinary samples including stool and biopsy samples.

[0036] In the particular case of *Giardia* and *Cryptosporidium*, the present invention provides methodologies for rapid, specific and high throughput screening, using real-time PCR or other sequence-based hybridization methodologies. This enables examination of large numbers of samples to identify asymptomatic individuals shedding cysts/oocysts, providing the true prevalence of parasitaemia in communities. Additionally, simultaneous genotyping capabilities as herein provided allow for predictive epidemiology, critical for action in outbreak situations.

[0037] It will be appreciated that “real-time PCR” is distinguished from endpoint (standard) PCR in that measurements are made during DNA amplification and are done so in real-time. Standard or endpoint PCR is measured at the end of a run, is not quantitative, and may take 1 plus days to obtain results. In real-time PCR, a sequence-specific primer set and a fluorescently labeled sequence-specific probe are used for detection of a specific target. The probes utilize the 5' exonuclease function of *Taq* DNA polymerase to cleave the fluorophore from the probe when bound to its target. Fluorescence is recorded over time as it accumulates with PCR cycling and it is directly proportional to the starting number of target copies in the initial sample. Real-time PCR provides accurate quantification of the target, as the target is quantified while amplification is still in the exponential part of the reaction. With multiplex real-time PCR, applied in embodiments of the present invention, the reporter dye for each target is detected simultaneously from each PCR reaction by a distinct emission wavelength (colour) after excitation by a light source. A real-time PCR diagnostics approach offers a wide concentration range in which it can detect the target organism (over 7 log units). This assay is also very sensitive, potentially detecting down to 1 copy of the target gene.

[0038] Embodiments of the present invention are now described in the examples which follow, and with reference to the accompanying drawings in which:

#### Brief Description of the Drawings

[0039] **Figure 1:** Range of bacterial detection in real-time PCR as shown by amplification plots. In the multiplex plot *lacZ* amplification is represented by black lines and closed circles, and *eae* amplification is represented by grey ‘x’s. The lines represent amplification of 10-fold serial dilutions of genomic DNA.

[0040] **Figure 2:** Standard curves generated from real-time PCR correspond to the amplification plots in Figure 1. The standard curve is generated of 10-fold serial dilutions of genomic DNA standards (closed squares) from  $1 \times 10^7$  to  $1 \times 10^0$  copies of *eaeA*/μl and  $2 \times 10^7$  to  $2 \times 10^0$  copies of *lacZ*/μl and shows sample starting concentration (open squares).

[0041] **Figure 3:** Range of protozoan detection in real-time PCR as shown by amplification plots. *G. lamblia* was detected using the  $\beta$ -giardin P241 primer/probe set and *C. parvum* by the COWP gene. The  $\beta$ -giardin and COWP plots demonstrate 10-fold serial dilutions and 2-fold serial dilutions were used to generate the multiplex amplification plot.

[0042] **Figure 4:** Standard curves generated from real-time PCR correspond to the amplification plots in Figure 3. In panel 1 ( $\beta$ -giardin) 10 fold serial dilutions ranging from 25 ng to 25fg of DNA corresponds to  $1.3 \times 10^5$  to 1 cyst. The standard curve for the COWP gene represents 10 fold serial dilutions of *C. parvum* DNA, from 5.7 ng to 5.7 fg and correspond to  $1 \times 10^5$  to 1 oocyst. The multiplex standard curves were generated from 2 fold dilutions of DNA ranging from 2.5 ng to 390 fg.

#### Detailed Description of the Invention

### **EXAMPLES**

[0043] Detailed descriptions of the methods used for detecting these organisms using real-time PCR are provided in the following examples. Differences in size and abundance in environmental samples between the 4 pathogens described herein necessitated the development and utilization of a variety of methods for collection and concentration of the pathogens from samples. For example, bacteria were enumerated on 100 ml water samples using a 0.2 um pore size filters due to their small size whereas, 2L water samples were concentrated for detection of protozoa and 1 to 3 um pore size filters employed. Similarly, the variation in hardness of the cell wall of these organisms necessitated the use of different DNA extraction methods for efficient DNA extraction.

### **EXAMPLE 1**

#### **Bacterial Strains and Culture Conditions**

[0044] The bacterial strains and isolates of protozoans used and the culture conditions are listed below.

[0045] *E. coli* (ATCC 8739) were cultured nutrient broth and incubated at 37°C, overnight on a rotary shaker (New Brunswick Scientific Co.) at 200 rpm, or maintained on nutrient agar (2%) plates. Cell population densities were quantified with a spectrophotometer (DU-64; Beckman) at 550 nm.

[0046] *E. coli O157:H7* (ATCC 35150, Oxoid Inc.) were maintained on tryptic soy agar. *E. coli* O157:H7 was cultured overnight at 37°C on a shaker in tryptic soy broth (TSB) and for selective identification on Sorbitol MacConkey Agar containing cefeximine and tellurite (CT-SMAC; Oxoid) at 37°C for 24 hours.

[0047] *M. aeruginosa* cultures (UTCC 300, 468, and 459) were maintained in liquid BG-11 medium (Rippka et al., 1979) at 25°C on a shaker (150 rpm) under a fluorescent light source 25-30  $\mu\text{Einm}^{-2} \text{ s}^{-1}$ . Strains were subcultured every two weeks. Cell population densities were quantified with a spectrophotometer (DU-64; Beckman) at 730 nm.

#### Protozoa:

[0048] ***Giardia* cysts:** Live *G. lamblia* cysts, produced by passage of the human strain CH3 of *G. intestinalis* through Mongolian gerbils, were purchased from Waterborne Inc. (New Orleans, LA). Cysts were delivered in PBS containing antibiotics, stored at 4°C and used within 1 month. The WB strain was obtained Dept. Biology, University of Alberta. The GA strain was obtained by extraction of DNA from cysts obtained from fecal sample of a patient in Ontario, Canada. *G. muris* Roberts-Thompson strain obtained from Waterborne Inc.

[0049] ***Cryptosporidium* oocysts:** Live *C. parvum* oocysts (IOWA strain) produced by passage in calves were purchased from Waterborne Inc., delivered in PBS containing antibiotics, stored at 4°C and used within 3 months. Live oocysts of the GCH1 isolate were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: contributed by Dr. Saul Tzipori.

## **EXAMPLE 2**

### **Collection and Concentration from Water Samples**

[0050] The methodologies for optimal collection and concentration of *E. coli*, *M. aeruginosa*, *G. lamblia*, and *C. parvum* are organism dependent.

[0051] *E. coli* (and coliforms) and Microcystis:

[0052] Collection from Water: Water samples were examined for the presence of *E. coli* and Microcystis. Environmental samples were collected in wide mouth 500ml polypropylene bottles (VWR, Mississauga, ON). Collected environmental (100ml) and bottled water (100-500ml) samples were concentrated onto 0.2 µm membranes (47 mm Supor™, Pall Gelman, Mississauga, ON) by vacuum filtration in Nalgene® filter units with receivers (model 300-4000; VWR, Mississauga, ON). In each experiment filtered MQ water was processed as a negative control and bacterially spiked water samples were processed as positive controls.

[0053] Collection and concentration of bacteria from Sponges: Sponges were placed into sterile bags and 50 ml of ddH<sub>2</sub>O containing 0.2% of Tween 20 was added to each bag. The bags were pulsified for 15 sec in a Pulsifier (Microbiology International). The homogenates were concentrated onto 0.2 µm membranes (47 mm Supor™, Pall Gelman, Mississauga, ON) by vacuum filtration in Nalgene® filter units with receivers (model 300-4000; VWR, Mississauga, ON). The sponges in the bag were washed two times using 50 ml of ddH<sub>2</sub>O by rigorous shaking and each wash was concentrated onto the filters. DNA was extracted from the filters using the procedure described in example 3.

[0054] Collection and concentration of bacteria from sponge swabs after growth in enrichment media: Sponges inoculated with *E. coli* were placed in 125 ml of nutrient broth or Tryptic soy broth (TSB) in wide mouth 500ml polypropylene bottles (VWR, Mississauga, ON) and were left on a shaker for 2 to 5 hr, at 37°C . Enriched media samples (25-35ml) were concentrated onto 0.2 µm membranes (47 mm Supor™, Pall Gelman, Mississauga, ON) by vacuum filtration in Nalgene® filter units with receivers (model 300-4000; VWR, Mississauga, ON). Tween 20 (0.25%) was added to the

culture media before collecting on the supor membranes. For each 35 ml of media concentrated on the filter, the filter was washed with 25 ml of 25% ETOH followed by 100ml of water. In each experiment filtered MQ water was processed as a negative control and bacterially spiked water samples were processed as positive controls. DNA was extracted from the filters using the procedure described in example 3.

#### ***Giardia* and *Cryptosporidium* :**

[0055] **Vacuum Filtration:** Water samples were collected in 10 L plastic carboys (Cole Palmer, Chicago, IL) and stored at 4°C until use (same day). Samples (2 L) were filtered through 3 µm cellulose nitrate filters, 47 mm diameter (Sartorius, Goettingen, Germany) in a parabolic stainless funnel (Gelman, Ann Arbor, MI) using a vacuum pressure between 10-15 PSI generated by a Millipore Vacuum/Pressure pump (115V,60 Hz; Millipore,). Following filtration of the sample, the funnel was rinsed with double-distilled (dd) water. Cellulose acetate filters, with a pore size of 1.2 µm were used for collection of *C. parvum* by vacuum filtration. For simultaneous detection of *Giardia* and *Cryptosporidium* from a single sample the sample was filtered through a 3 µm cellulose nitrate filter (as described above) and the filtrate was filtered through a 1.2 µm cellulose acetate filter.

### **EXAMPLE 3**

#### **DNA Extraction**

[0056] To evaluate the efficiency of DNA extraction for *E. coli*, *M. aeruginosa*, *G. lamblia*, and *C. parvum* different extraction procedures were evaluated for the different organisms and different types of samples. The commonly adopted methods are described below.

[0057] ***E. coli* (and coliforms):** DNA extraction membranes from the collection units, described above in example 2 was aseptically transferred into a 2 ml screw-cap microfuge tube and 200 µl of PrepMan™Ultra (ABI, Foster City, CA) was added and the tube was vortexed to disperse the sample. The sample was then heated to 100°C in a water bath for 10 min. The samples were removed and allowed to cool for 2 min, then briefly centrifuged to transfer the supernatant to a clean microfuge tube. This one

step procedure allows use of the extract directly in the 5' nuclease real-time PCR reactions.

[0058] ***Microcystis*:** DNA extraction membranes were aseptically transferred to a 1.5 ml microfuge tube from the filtration units. The DNeasy Tissue kit (Qiagen, Mississauga, ON) was used for DNA extraction from the cells on the membrane, using a modified method DNA extraction from Gram negative bacteria. The membrane was suspended in 360 $\mu$ l ATL buffer and 40 $\mu$ l Proteinase K, vortexed and incubated at 55°C for 1hr to overnight. The sample was vortexed for 15 sec, and 400 $\mu$ l of AL buffer was added. The sample was vortexed again and incubated at 70 °C for 10 min, 400 $\mu$ l of absolute ethanol was added the sample was vortexed again. The manufacturer's protocol was followed onward and DNA was eluted in two steps with 50  $\mu$ l AE buffer.

***Giardia* and *Cryptosporidium*:**

[0059] **DNeasy Kit**: DNA was extracted from cysts/oocysts using the DNeasy Tissue kit (Qiagen, Hilden, Germany). A modification of the animal tissue protocol was employed: 1). Tubes containing the pellet of cysts or oocysts were taped to dislodge the cells, suspended in 180 $\mu$ l ATL plus 20 $\mu$ l of Proteinase K and incubated for 1 hr in a 56°C water bath; 2) cells were subjected to 3 cycles of freeze/thaw, each cycle consisting of 2 min each in liquid nitrogen followed by boiling water; 3). 3 bursts of sonication, each of 20 sec duration, using a microprobe on a Model W-220F Cell Disruptor (ULTRASONICS INC) or alternatively, 30 min sonication in a 2-1/2'cup horn (Sonics and Materials Inc., Newtown, CT ), or 2 min vortex in the presence of 0.02 gm of 425-600 $\mu$ m glass beads (Sigma, St. Louis, MO). DNA was quantified using the PicoGreen® dsDNA quantitation reagent (Molecular Probes, Seattle, WA). The manufacturer's protocol volumes were reduced to obtain a 50 $\mu$ l total reaction volume and 10 $\mu$ l of sample was added to each well. Fluorescence was determined using the FAM filter set in an Mx4000 (Stratagene). The use of the DNeasy kit with freeze/thaw and sonication yielded 100% efficient extraction of DNA based on comparison of DNA concentration measured by PicoGreen, compared with the theoretical yield of DNA/cyst or oocyst.

**[0060] Extraction of DNA from filters following concentration of environmental water samples:** Cellulose nitrate and cellulose acetate filters were removed, folded twice, lengthwise with the upper surface facing out and placed into Eppendorf tubes. DNA was extracted directly from the filter using the DNeasy kit (Qiagen). Following incubation in 180 µl ATL and 20 µl proteinase K for 1 hr at 56°C the filter was washed with 200 µl of ATL and the wash pooled with the initial cell lysate. The procedure outlined in example above was followed to extract DNA from the cells. DNA was eluted from the column using either 1 round of 50µl dd water or 2 rounds of 50µl dd water.

**[0061] Extraction of *Giardia* DNA from stool:** DNA was extracted from stool using the QIAamp® DNA stool kit (Qiagen) with modifications. An aliquot of 0.2 gm of SAF-fixed stool was washed twice in sterile phosphate-buffered saline, pH 7.2 (PBS), by centrifugation at 12,000xg for 10 min. The supernatant was removed and the pellet was suspended in 0.6 ml of ATL buffer (Qiagen, Germany) and incubated in a 56°C water bath for 4 hr. The sample was subjected to 3 cycles of freeze/thaw (as described above) and incubated at 56°C overnight. After three, 20 sec bursts of sonication, an additional 0.6 ml ATL was added to each tube, the contents mixed by vortex for 15 sec and split equally into two tubes. Half an inhibitex tablet was added to each tube containing sample and the manufacturer's procedure for the QIAamp® DNA stool kit (Qiagen) was followed. DNA was eluted from the silica gel column using 2 rounds of 100µl sterile, dd water. Samples were stored at –20°C until use.

**[0062] Extraction of *Giardia* and *Cryptosporidium* from raw sewage:** One L raw sewage samples were centrifuged at 3,000 xg for 30 min to pellet cells. DNA was extracted directly from the pellet by the following method. The pellets were resuspended in ATL lysis buffer and proteinase K and inhibitor removers were added to the sample: Chelex® (BIO RAD) slurry, to a final concentration of 20% and PVP-360 (ICN, Aurora, OH), to a final concentration of 2%. The samples were incubated for 30 min at 56°C, subjected to freeze/thaw and sonication and centrifuged at 12,000xg for 10 min. The supernatant was processed on two DNeasy columns following the manufacturer's description and eluted from the column using 2 volumes of 50µl of dd water. The samples were pooled to equal a total volume of 200 µl.

## EXAMPLE 4

### Oligonucleotide Design

[0063] Upon selection of a gene of interest to serve as a target for 5' nuclease PCR, subsets of the target gene were selected as regions for oligonucleotide design based on regions of low homology to other targets from a blastn search (NCBI). From subsets of blastn hits, regions that showed high homology to other microorganisms, especially those likely to be found in water, food, or clinical samples were excluded. The gene domains with the lowest levels of homology were used in Primer Express Software (ABI) that generated an output list of 200 possible primer/probe combinations the list was refined and regenerated for a specific oligonucleotide within a set until the desired parameters were met. From the generated oligonucleotide combinations, selections were based on %GC content, GC relative distribution, strings of identical nucleotides, secondary structure, and Tm. All selected oligonucleotides were subjected to a blastn analysis on GenBank (NCBI) prior to synthesis, to ensure specificity for detection of the target organism. Primers and probes were synthesized using standard methodology. The probes were 5' labeled with either FAM (6-carboxyfluorescein,  $\lambda_{em}=518\text{nm}$ ), HEX (5'-Hexachloro-Fluorescein,  $\lambda_{em}=553\text{ nm}$ ), JOE (6-carboxy-4', 5'-dichloro-2', 7'-dimethoxyfluorescein,  $\lambda_{em}=548\text{ nm}$ ) or Cy5 (1-(epsilon-carboxypentyl)-1'-ethyl-3,3,3',3'-tetramethylindodicarbocyanine-5,  $\lambda_{em}= 667\text{ nm}$ ); both probes were also 3' labeled with a non-fluorescent Black Hole Quencher (BHQ) dye (Biosearch Technologies Inc.; IDT Technologies).

[0064] *E. coli* (and coliforms): A *lacZ* (GenBank Acc # V00296) primer and probe set was designed to detect the beta-galactosidase gene, and recognizes both total coliforms (including non-toxigenic *E. coli* and the toxigenic strain, *E. coli* O157:H7. A general indicator that would encompass coliform bacteria is *lacZ*, encoding the enzyme  $\beta$ -D-galactosidase, which is present in all coliforms (Apte et al., 1995), including *E. coli* O157:H7.

[0065] *E. coli* O157:H7: We have also designed an *eaeA* primer set and probe to detect the 3' end of the attaching and effacing gene, encoding intimin, (GenBank Acc # X60439) of *E. coli* O157:H7.

[0066] *Microcystis aeruginosa*: To distinguish between toxic-microcystin producing cyanobacteria and non-toxic forms, the MISY primer set was designed to amplify a region of the *mcyA* (GenBank Acc #AB019578) gene from the microcystin synthetase gene operon, involved in the synthesis of the microcystin toxin (122 bp amplicon). *McyA* is directly involved in biosynthesis of the toxin, and disruption mutants do not produce detectable levels of microcystins (Tillett et al., 2000). *McyA* is part of the peptide synthetase module of the microcystin synthetase gene operon, insertional mutagenesis into this gene abolished toxin production (Nishizawa et al., 2000).

[0067] These *mcyA* primers were found to be specific to toxic strains of *M. aeruginosa* and did not yield any amplification products from any of the other cyanobacterial or eubacterial species examined (*M. aeruginosa* (strains UTCC 300, UTCC 459, UTCC 468, and PCC7005), *A. flos-aquae* (strains AF67 and AF64); non-toxigenic *E. coli* (ECUTM), *Bacillus subtilis* (UTM 206), *Proteus vulgaris* (BCC 219), and *Enterobacter aerogenes* (BCC 208)). The 5' nuclease PCR results discriminated between toxic strains of *M. aeruginosa* (MA459, MA300) and a non-toxic strain (MA468). There was no increase in fluorescence detection above background for non-toxic MA468 samples in real-time PCR experiments (Ct of 40).

[0068] *G. lamblia*: Two primer/probe sets were designed against the complete coding sequence of the β-giardin gene (GenBank Accession #M36728) of the Portland-1 strain of *G. lamblia* (Holberton et al., 1995). This gene codes for a structural protein that is a component of the adhesive disk of the parasite, important in binding of trophozoites to the intestinal epithelium of their host. Two distinct primer/probe sets were designed, the first primer set P241 was based on the region 222-296 and the second set, P434, was based on region 411-485 of β-giardin (GenBank Accession #M36728) (Table 2).

[0069] *C. parvum*: The *Cryptosporidium* oocyst wall protein (COWP) (GenBank Accession #Z22537) was selected as the target gene for designing the primer probe set for detection of *C. parvum*. This gene was selected because it codes for a protein that is important in maintaining the integrity of the oocyst wall allowing the parasite to withstand harsh environmental factors until ingested by a new host. In designing the sequences, 26 partial sequences coding for the oocyst wall protein, from different

isolates and species of *Cryptosporidium* were examined to identify regions of the gene specific to *C. parvum* and to specific genotypes 1 and 2 of *C. parvum*. These sequences were entered into the BIMAS www READSEQ Sequence Conversion program for conversion into a format readable by ClustalW. The converted sequences were entered into the ClustalW program (European Bioinformatics Institute) and a multiple alignment performed to identify regions of the gene. The sequences and their GenBank Accession #'s are as follows: *C. parvum* CBAHI (#AJ310765), *C. baleyi* (#AF266276), *C.spp*715-dog (#AF266274), *C. felis* (#AF266263), *C. spp*815-bullsnake (#AF266277), *C. meleagridis* (#AF248742), *C. meleagridis* (AF266266), *C. wrairi* (#AF266271), *C. wrairi* (U35027), *C. parvum* G2 (#AF248743), *C. parvum* CPACH-1 (#AJ310766), *C. spp*6-bovine (#AF266273), *C. parvum* G2 (#AF161577), *C. spp* 4A-mouse (#AF266268), *C. spp*-monkey (#AF266272), *C. parvum* G1 (#AF248741), *C. parvum* 181 (#AF266265), *C. parvum* G1 (#AF161578), *C. spp* 351-ferret (#AF266267), *C. spp* 428-kangaroo (#AF266269), *C. spp* 499-pig (#AF266270), *C. serpentis* (#AF266275), *C. serpentis* (#AF161580), *C. andersoni* (#AAF266262), *C. muris* (#AF266264) and *C. muris* (#AF161579).

[0070] The region selected for *C. parvum* detection ranged from 583-733 of the coding sequence of the COWP gene (GenBank Acc.#Z22537).

**TABLE 1.** Primer and Probe sequences.

| Target                | Oligo <sup>†</sup> | Sequence (5' to 3')           | SEQ ID | Location within gene (CDS) | Amplicon Size (bp) |
|-----------------------|--------------------|-------------------------------|--------|----------------------------|--------------------|
| <i>eaeA</i>           | F                  | aataactgcttggattaaacagacatct  | 1      | 2673-2700                  | 87                 |
|                       | R                  | ggaaggagggtttgtttaggtt        | 2      | 2734-2759                  |                    |
|                       | P                  | aagtgcgtgatactccagaacgctgc    | 3      | 2703-2731                  |                    |
| <i>lacZ</i>           | F                  | ggatctgcattgtcagacatg         | 4      | 2754-2775                  | 142                |
|                       | R                  | ctgttgactgttagccgtgtat        | 5      | 2874-2895                  |                    |
|                       | P                  | taccccgtaacgtctcccgagcg       | 6      | 2778-2800                  |                    |
| <i>mcyA</i>           | F                  | cgaccgaggaaattcaagct          | 7      | 1438-1457                  | 122                |
|                       | R                  | agtatccgaccaagttacccaaac      | 8      | 1536-1559                  |                    |
|                       | P                  | ttaaatcggaaattcccgagaaaatgcgt | 9      | 1459-1489                  |                    |
| $\beta$ -giardin P241 | F                  | catccgcgaggaggc               | 10     | 222-239                    | 74                 |
|                       | R                  | gcagccatgggtgcgtatct          | 11     | 296-278                    |                    |
|                       | P                  | aagtccgcgacaacatgtacctaacga   | 12     | 241-268                    |                    |
| $\beta$ -giardin P434 | F                  | cctcaagagccgtgaacgtatctc      | 13     | 411-432                    | 74                 |
|                       | R                  | agctggtcgtacatcttcctt         | 14     | 485-462                    |                    |
|                       | P                  | ttctccgtggcaatggccgt          | 15     | 434-455                    |                    |
| COWP                  | F                  | caaattgtatccgtttgtcctctg      | 16     | 583-607                    | 150                |
|                       | R                  | ggcatgtcgattctaattcagct       | 17     | 733-711                    |                    |
|                       | P                  | tgcatacatgttgtccgtacaaattgaat | 18     | 702-672                    |                    |

<sup>†</sup>Forward (F) and reverse (R) primers and dual-labeled hydrolysis probe (P); the probe for *eaeA* was 5' labeled with JOE, the *lacZ* probe was 5' labeled with FAM, the  $\beta$ -giardin probes P241 and P434 were FAM labeled and the COWP probe was labeled with HEX. Probes were 3' quenched with TAMRA or BHQ-1 (Biosearch Technologies, Inc.). CDS= coding sequence.

## EXAMPLE 5

### Real-time PCR Conditions

[0071] Real-time (5'nuclease) PCR reactions were carried out using reagents from the Brilliant™ qPCR kit (Stratagene, La Jolla, CA). Each reaction contained 4 mM MgCl<sub>2</sub>, 800 nM dNTPs, 8% glycerol, 0-100 $\mu$ g/ml BSA, 20 nM ROX (6-carboxy-X-rohamidine) normalizing dye, 1.25 U SureStart *Taq* DNA polymerase, 200 nM probe, 300-900 nM (Table 3) of each primer, and 1-10  $\mu$ l template in a 25  $\mu$ l reaction. Alternatively, for samples known to contain a low concentration of target DNA, reaction volumes were increased to 50 or 100 $\mu$ l to allow addition of larger volumes of template. Reactions were carried out in an Mx4000 (Stratagene), with a 10 min incubation at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Three fluorescence readings were collected at the end of each 60°C cycle. Each sample was

run in triplicate and data analyzed using the Mx4000 software (Stratagene). Similar results were obtained when the reactions were performed in an SDS 7700 (ABI).

**Table 2. Final concentration of oligonucleotides in real-time PCR reactions**

| Target                | Oligo <sup>†</sup> | Working Concentration (nM) |
|-----------------------|--------------------|----------------------------|
| <i>eaeA</i>           | F                  | 900                        |
|                       | R                  | 900                        |
|                       | P                  | 200                        |
| <i>lacZ</i>           | F                  | 300                        |
|                       | R                  | 300                        |
|                       | P                  | 200                        |
| <i>mcyA</i>           | F                  | 50                         |
|                       | R                  | 300                        |
|                       | P                  | 200                        |
| $\beta$ -giardin P241 | F                  | 600                        |
|                       | R                  | 300                        |
|                       | P                  | 200                        |
| $\beta$ -giardin P434 | F                  | 300                        |
|                       | R                  | 300                        |
|                       | P                  | 200                        |
| COWP                  | F                  | 300                        |
|                       | R                  | 300                        |
|                       | P                  | 200                        |

**Elimination of *E. coli* DNA contamination of Taq reagent:**

[0072] Currently, commercial Taq polymerases are produced as recombinant proteins in *E. coli* and contain low levels of *E. coli* DNA ( $\leq$ 1pg of DNA, personal communication Stratagene). When used in qPCR detection of the *LacZ* gene of *E. coli*, the negative controls produce Ct values due to the bacterial DNA contamination of certain lots of the Taq reagent. These numbers mask the qPCR detection of 1,000 or fewer *E. coli* in the samples. For this reason contaminating DNA will be destroyed using restriction enzyme digestion.

[0073] To remove DNA contamination from the Taq polymerase, the polymerase was subjected to Mbo II digestion. There is one Mbo II cutting site in the middle of the *LacZ* probe sequence. An aliquot of 1 ul containing 5 Units of Mbo II was added to the qPCR master mix containing the 10x buffer, water, dNTPs and Taq polymerase.

The sample was incubated for 15 min at 37°C followed by inactivation of Mbo II at 95°C for 5min. Once cooled, the primers, probe, reference dye and glycerol were added to the master mix and the qPCR assay was performed.

[0074] Mbo II treatment removed the Ct values in negative controls for *LacZ* detection (Table H). Temperature treatment of the master mix did not alter the detection compared with no treatment (not shown). There was a 1-log reduction in detection of spiked DNA ( $5 \times 10^4$  copies to  $5 \times 10^1$  copies) following Mbo II treatment (Table H). No Ct values were observed in the negative controls when detecting the *eaeA* gene for the toxigenic *E. coli* 0157:H7 in the qPCR assay. There is one Mbo II restriction site in the reverse primer region of the *eaeA* amplicon. Digestion of Taq polymerase using Mbo II and inactivation of the enzyme prior to the qPCR assay did not significantly alter detection of the *eaeA* target.

[0075] Restriction digestion of Taq polymerase using Mbo II will be used whenever commercial lots of Taq polymerase contain DNA that is measurable in the qPCR assay for detection of the *LacZ* gene of coliforms.

Table 3: Mbo II Treatment of Taq Polymerase for qPCR Detection of *LacZ* and *eaeA*.

| PCR Template           | Cycle Threshold (Ct) |            |             |            |
|------------------------|----------------------|------------|-------------|------------|
|                        | <i>LacZ</i>          |            | <i>eaeA</i> |            |
|                        | No Mbo II            | Mbo II     | No Mbo II   | Mbo II     |
| ddH <sub>2</sub> O     | 38.47±0.94           | No Ct      | No Ct       | No Ct      |
| -ve Filter*            | 34.78±0.53           | No Ct      | No Ct       | No Ct      |
| $5 \times 10^4$ copies | 22.41±0.43           | 28.78±0.56 | 20.60±0.33  | 21.61±0.25 |
| $5 \times 10^3$ copies | 27.07±0.71           | 34.59±0.67 | 24.50±0.33  | 25.57±0.36 |
| $5 \times 10^2$ copies | 31.86±0.19           | 38.70±1.49 | 28.69±0.02  | 29.32±0.31 |
| $5 \times 10^1$ copies | 36.05±0.26           | No Ct      | 31.79±1.15  | 32.18±0.54 |

\*--ve Filter, extraction of a filter treated with water only.

The ddH<sub>2</sub>O and -ve Filter templates were used as negative controls.

Copies of *E. coli* and *E. coli* 0157:H7 DNA for detection of the *LacZ* and *eaeA*, respectively.

## EXAMPLE 6

### Sensitivity and Specificity of real-time primer/probe oligonucleotides

#### *E. coli*

#### *Microcystis aeruginosa:*

#### *Giardia and Cryptosporidium:*

[0076] The β-giardin P241 and P434 primer/probe sets were very sensitive in detecting DNA extracted from *Giardia* cysts and detected DNA across a broad range of dilutions 7 logs, from as few as 1 cyst to as many as  $5 \times 10^5$  (Figure 3 and 4). Detection of *C. parvum* oocysts was in the same range, with the capability of detecting 2 oocysts. Detection of higher concentrations of *Giardia* and *Cryptosporidium* is possible when using larger starting number of cells in the DNA extraction. The primer/probe sets did not detect other unrelated sources of DNA (eg. *E. coli*, *O. novo ulmi*) in real-time PCR demonstrating specificity to the organisms they were designed to detect (Table 4). Probe 241 detects both *G. lamblia* and *G. muris* whereas P434 detected *G. lamblia* only.

Table 4. Specificity test of Oligonucleotides by Endpoint or Real-time PCR

| DNA Source <sup>a</sup>            | <i>E. coli</i> <sup>b</sup> | <i>E. coli</i><br>O157:H7 <sup>b</sup> | <i>M. aeruginosa</i> <sup>b</sup> | <i>G. lamblia</i><br>P241 <sup>c</sup> | <i>G. lamblia</i><br>P434 <sup>c</sup> | <i>C. parvum</i> <sup>c</sup> |
|------------------------------------|-----------------------------|--|-----------------------------------|--|--|-------------------------------|
| <i>A. flos-aquae</i><br>(AF64)     | -                           | -                                      | -                                 | nd                                     | nd                                     | nd                            |
| <i>A. flos-aquae</i><br>(AF67)     | -                           | -                                      | -                                 | nd                                     | nd                                     | nd                            |
| <i>B. cereus</i>                   | -                           | -                                      | -                                 | nd                                     | nd                                     | nd                            |
| <i>B. subtilis</i>                 | -                           | -                                      | -                                 | nd                                     | nd                                     | nd                            |
| <i>C. parvum</i>                   | nd                          | nd                                     | nd                                | -                                      | -                                      | +                             |
| <i>E. aerogenes</i>                | nd                          | nd                                     | -                                 | nd                                     | nd                                     | nd                            |
| <i>E. coli</i> (ATCC<br>8739)      | +                           | -                                      | -                                 | -                                      | -                                      | -                             |
| <i>E. coli</i> O157:H7             | +                           | +                                      | nd                                | nd                                     | nd                                     | nd                            |
| <i>G. lamblia</i> H3               | nd                          | nd                                     | nd                                | +                                      | +                                      | -                             |
| <i>G. lamblia</i> WB               | nd                          | nd                                     | nd                                | +                                      | +                                      | -                             |
| <i>G. muris</i>                    | nd                          | nd                                     | nd                                | +                                      | -                                      | -                             |
| <i>M. aeruginosa</i><br>(UTCC 300) | -                           | -                                      | + <sup>b,c</sup>                  | -                                      | -                                      | -                             |
| <i>M. aeruginosa</i>               | -                           | -                                      | - <sup>b,c</sup>                  | nd                                     | nd                                     | nd                            |

|                                    |    |    |                  |    |    |    |
|------------------------------------|----|----|------------------|----|----|----|
| (UTCC 468)                         |    |    |                  |    |    |    |
| <i>M. aeruginosa</i><br>(UTCC 459) | -  | -  | + <sup>b,c</sup> | nd | nd | nd |
| <i>M. aeruginosa</i><br>(PCC 7005) | -  | -  | -                | nd | nd | nd |
| <i>M. aeruginosa</i><br>(PCC 7806) | -  | -  | nd               | nd | nd | nd |
| <i>O.novo- ulmi</i><br>(VA30)      | nd | nd | nd               | -  | -  | -  |
| <i>P. vulgaris</i>                 | -  | -  | -                | nd | nd | nd |

nd= not determined

<sup>a</sup> DNA from *Anabena flos-aquae* (AF 64 and AF 67), *Bacillus cereus*, *Bacillus subtilis*, *Enterobacter aerogenes* (Brock Culture Collection, BCC 208), *Escherichia coli* (ATCC 8739), *Enterobacter aerogenes* (Brock Culture Collection, BCC 208), *Escherichia coli* O157:H7 (ATCC 35150), *Giardia lamblia* (H3 and WB), *Giardia muris* (Roberts-Thompson strain), *Microcystis aeruginosa* (strains UTCC 300, UTCC 459, UTCC 468, Pasteur Culture Collection (PCC 7005 and PCC7806), *Ophiosoma novo-ulmi*, and *Proteus vulgaris* (BCC 219)

<sup>b</sup> Specificity of primers as detected by amplification of specific fragment in endpoint PCR.

<sup>c</sup> Specificity of primers and probe as detected by emission of fluorescence in real-time PCR.

## EXAMPLE 7

### Standard curves for Quantitation of Pathogenic Organisms

[0077] To enable quantitation of cells per sample, standard curves were generated for all 4 target organisms (Figures 2 and 4).

#### *E. coli*

[0078] Cell cultures were divided into 1 to 1.5 ml aliquots for DNA extraction with the DNeasy Tissue Kit (Qiagen). The manufacturer's protocol for extraction from Gram negative bacteria was followed, and elution was performed with 20 mM Tris-HCl in two steps of 25 to 50 µl each. The DNA was serially diluted and used to generate the standard curve (see example 5, real-time PCR).

[0079] Standard curves were constructed from *E. coli* genomic DNA of a known concentration, as determined spectrophotometrically (OD<sub>260</sub>). The gene copy number, for *lacZ* or *eaeA*, was calculated based on the genome sizes of *E. coli* (4.6 Mb) and *E.*

*coli* O157:H7 (5.5 Mb), respectively (GenBank); with *lacZ* and *eaeA* as single copy genes. The calculation was based on the following equation:

$$\text{[DNA, g/ml]} \times 6.0221367 \times 10^{23} \text{ gene copies/mol}$$

$$\text{genome size, bp} \times 2 \text{ b/bp} \times 330 \text{ g/mol/b}$$

where b= base, and bp=base pair. Standards ranged from  $1 \times 10^7$  gene copies/ $\mu\text{l}$  ( $5 \mu\text{l}$  of template were added each  $25 \mu\text{l}$  reaction) to  $1 \times 10^0$  copies/ $\mu\text{l}$ , as obtained by 10-fold serial dilutions. DNA was also extracted (as above) from samples spiked with different relative concentrations of each bacterial strain (unknowns), to obtain quantitative results on the starting concentration of each type of *E. coli* in the unknown samples. Each sample was run in triplicate and a no template control was used in each PCR run.

#### **Protozoa:**

[0080] Standard curves were generated using serial dilutions (10, 5 and 2 fold dilutions) of DNA purified from cysts/oocysts, using the maximum efficiency (100%) method of extraction (DNeasy with freeze/thaw and sonication) and Picogreen dsDNA quantitation. Both the  $\beta$ -giardin and COWP genes are expressed as single copy genes within the nuclei. Cysts of *Giardia* contain 2 trophozoites that have undergone multiple steps of nuclear division and thus 16 copies of total genetic information are contained within each cyst (Bernander et al., 2001). Within *Cryptosporidium* oocysts are 4 nucleated sporozoites. Therefore, there are 16 copies of the  $\beta$ -giardin gene available in each *Giardia* cyst and 4 copies of the COWP gene per oocyst. The total genome sizes are 12 MB and 10.4 MB, for *Giardia* and *Cryptosporidium*, respectively.

[0081] Using the conversion: Mass (pg) = bp/ $0.9869 \times 10^9$ . The DNA mass of *Giardia* is 0.195 pg/cyst and is 0.04 pg/oocyst for *Cryptosporidium*.

## **EXAMPLE 8**

### **Multiplex Assays**

[0082] Multiplex assays for detection of 2 or more organisms in one sample significantly reduce the labour and supply costs when performing large numbers of samples. Described herein are 2 multiplex assays using sequence-specific primer/probe sets.

#### *E. coli*

[0083] The probes for the *lacZ* and *eaeA* gene targets have been labeled with different fluorogenic probes (FAM and JOE, respectively), and can successfully identify both the toxigenic and non-toxigenic forms of *E. coli* in the same reaction run (Figures 1 and 2).

#### *G. lamblia* and *C. parvum*

[0084] A multiplex real-time PCR assay using  $\beta$ -giardin (FAM-labeled) and COWP (Hex- labeled) detected *G. lamblia* and *C. parvum* with equivalent sensitivities to a singleplex assay (see amplification plots and standard curves, Figures 3 & 4). Additionally, the amplicons generated by multiplex PCR were sequenced and proved to be identical to amplicons generated in the singleplex PCR.

## **EXAMPLE 9**

### **Real world application of real-time PCR to detection of *E. coli* in water**

[0085] We have applied real-time PCR to the detection of *E. coli* in lake water (Table 5) and bottled drinking water (Table 6).

TABLE 5. Comparison of total *E. coli* cells/100ml measurements from Heart and Professor's Lake in Peel Region, Ontario, obtained by culturing versus with 5'nuclease PCR on July 31, 2002.

| Site | MOH Plate Count <sup>a</sup><br>(cells/100ml) | UTM 5' Nuclease PCR <sup>b</sup><br>(cells/100ml) |
|------|---|---|
| 1A   | 220   | 165   |
| 2A   | 20  | 73  |
| 3A   | 20  | 111   |
| 4A   | 50  | 187   |
| 5A   | 20  | 72  |
| 6A   | 50  | 50  |
| 1B   | 60  | 128   |
| 2B   | 10  | 43  |
| 3B   | 10  | 128   |
| 4B   | 20  | 77  |

<sup>a</sup>Counts obtained from the Ontario Ministry of Health and Long Term Care (MOH) by culturing 10 ml of water from Heart and Professor's Lakes on media. Counts were rounded up to the nearest 10 cells/100ml.

<sup>b</sup>Counts obtained by performing multiplex 5'nuclease PCR (to detect total *E. coli* and toxigenic *E. coli* O157:H7, by amplification of *lacZ* and *eaeA*, respectively), by concentrating 100 ml of water from Heart and Professor's Lakes and extracting DNA prior to performing 5'nuclease PCR.

TABLE 6. Colony Growth and Endpoint and Real-Time PCR Quantification of total *E. coli* in Commercially Sold Bottled Water

| Bottled Water Brand | % Colony Growth | % Bottles with <i>LacZ</i> Amplification with Endpoint PCR <sup>b</sup> | % Bottles with <i>LacZ</i> Amplification with Real-Time PCR (fraction) | Real-time PCR Concentration Range (copies or cells/bottle) |
|---------------------|-----------------|---|--|--|
| E                   | 44              | 22  | 64<br>(7/11)   | 14.00±2.47—2.50±0.00                                       |
| F                   | 33              | 44  | 56<br>(5/9)  | 6.00±0.85—2.50±2.02  |
| G                   | 33              | 11  | 17<br>(2/12)   | 7.00±11.2—3.00±1.89  |
| H                   | 11              | 22  | 33<br>(4/12)   | 4.00±0.61—3.00±1.09  |

<sup>a</sup>Colony growth on LB solid media, with incubation for 24 hr at 37°C.

<sup>b</sup>21 bottles per brand were sampled from three different lot numbers

## EXAMPLE 10

### Protozoan Genotype determination

[0086] Primer and probe set P241 amplifies and detects all the strains of *G. lamblia* and the *G. muris* spp, whereas primer and probe set P434 is dependent on the sequence of the strain. Sequence variation within this region of the β-giardin gene (411-485) provides a means of genotyping *G. lamblia*. Oligonucleotides based on the coding sequence of the β-giardin gene of the Portland-1 strain of *G. lamblia* (GenBank Acc.# M36728) detect assemblage A isolates and oligonucleotides based on the H3 isolate sequence (sequenced in our lab) detect assemblage B (Table 7). These are specific to *G. lamblia* assemblages and do not detect *G. muris*, the murine species of *Giardia* (Table 8).

[0087] Use of molecular beacon probes targeting the COWP gene will discriminate between genotypes 1 and 2 of *C. parvum* based on single base pair mismatches.

Table 7. Specific sequences of *Giardia* genotyping primers and probes within the 411-485 bp region of the β-giardin gene.

| Oligo | β-giardin P434 Assemblage | Sequence (5' to 3')      | SEQ ID NO |
|-------|---------------------------|--------------------------|-----------|
| F     | A                         | cctcaagagcctgaacgatctc   | 13        |
|       | B                         | cctcaagagcctgaacgaccctc  | 19        |
| R     | A                         | agctggtcgtacatcttcttcctt | 14        |
|       | B                         | agctggtcatacatcttcttcctc | 20        |
| P     | A                         | ttctccgtggcaatgcccgtct   | 15        |
|       | B                         | ttctccgtggcgatgcctgtct   | 21        |

Forward (F); Reverse (R); Probe (P)

**TABLE 8. Genotype detection using  $\beta$ -giardin P434 compared to recognition of all *Giardia* tested by  $\beta$ -giardin P241.**

| Source of <i>Giardia</i> | Ct values with Specific Probes |                        |
|--------------------------|--------------------------------|------------------------|
|                          | P241                           | P434<br>(Assemblage A) |
| <i>G. lamblia</i>        |                                |                        |
|                          | WB                             | 28.11                  |
|                          | H3                             | 25.95                  |
|                          | G-A Stool Isolate              | 27.21                  |
| <i>G. muris</i>          |                                | 23.32                  |
|                          |                                | No Ct                  |

Assemblage A genotypes: WB, GA stool isolate

Assemblage B genotypes: H3

[0088] The p434 primer probe set was used to genotype the *Giardia* positive stool specimens into assemblage A and B (Table 9). The majority of the samples were of assemblage B, (human genotype) and three mixed infections of assemblages A and B were also observed (Table 9). The two major assemblages of *Giardia* were also detected in raw sewage samples; assemblage B was the predominant genotype (Table 10).

**Table 9: Major Genotype Detection of *G. lamblia* in Stool.**

| Stool Specimen | Number of Cysts           |                           |
|----------------|---------------------------|---------------------------|
|                | Assemblage A <sup>a</sup> | Assemblage B <sup>b</sup> |
| A              | 0                         | 11,558                    |
| B              | 6,331                     | 1,034                     |
| C              | 0                         | 1,428                     |
| D              | 0                         | 2,068                     |
| E              | 0                         | 27,218                    |
| F              | 69                        | 118,035                   |
| G              | 1,262                     | 0                         |
| H              | 0                         | 4,852                     |
| I              | 0                         | 3,916                     |
| J              | 40,530                    | 781                       |
| K              | 0                         | 34,081                    |
| L              | 0                         | 352                       |
| M              | 0                         | 456                       |
| N              | 5,593                     | 0                         |

<sup>a</sup> Detection of *Giardia* using the P434 P-1 (assemblage A) sequence of primers-probe.<sup>b</sup> Detection of *Giardia* using the P434 H3 (assemblage B) sequence of primers-probe.

**Table 10: Major Genotype Detection of *G. lamblia* in Raw Sewage.**

| Sample           | Number of <i>G. lamblia</i> Cysts |                           |
|------------------|-----------------------------------|---------------------------|
|                  | Assemblage A <sup>a</sup>         | Assemblage B <sup>b</sup> |
| Negative Control | 0                                 | 0                         |
| Auteuil 1        | 496                               | 5146                      |
| Auteuil 2        | 2476                              | 8340                      |
| Auteuil 3        | 5672                              | 7736                      |
| Fabreville 1     | 838                               | 1815                      |
| Fabreville 2     | 2196                              | 3663                      |
| Fabreville 3     | 545                               | 3331                      |

<sup>a</sup> Detection of *Giardia* using the P434 P-1 (assemblage A) sequence of primers-probe.<sup>b</sup> Detection of *Giardia* using the P434 H3 (assemblage B) sequence of primers-probe. The Auteuil and Fabreville treatment facilities, Laval, Quebec.

## **EXAMPLE 11**

### **Removal of PCR Inhibitors from Environmental Samples**

#### **PCR Inhibitor Removal:**

[0089] Concentration of 2 L water samples resulted in inhibition of real-time PCR. Addition of BSA (Fraction V, SIGMA) at a final concentration of 100µg/ml or milk powder at a concentration of 2mg/ml resulted in the removal of the inhibitors from 3 out of 4 water bodies tested. Samples from 1 lake were completely inhibitory to real-time PCR in the presence of BSA and required additional steps to remove inhibitors. Additional inhibition removal was carried out during concentration of water samples and DNA extraction. Following filtration of 2 L of water through the 3 µm cellulose nitrate filter, the filter was treated with 20 ml of 0.5 M EDTA pH 8.0 for 5 min then washed with dd water. After washing cysts/oocysts from the filter (described in example 3) the following inhibitor removers were added to the sample in ATL buffer: Chelex® (BIO RAD) slurry, to a final concentration of 20% and PVP-360 (ICN, Aurora, OH), to a final concentration of 2%. The samples were incubated for 30 min at 56°C, subjected to freeze/thaw and sonication and centrifuged at 12,000xg for 10

min. The supernatant was processed on a DNeasy column following the manufacturer's description and eluted from the column in 50µl of dd water.

[0090] To detect the presence of inhibitors, environmental sample extracts were spiked with a known concentration of DNA and the Ct values from real-time PCR were compared to the same concentration spiked into dd water (Table 11). The addition of BSA to the PCR mix was sufficient to remove inhibitors from concentrated Heart Lake water samples, enabling amplification of spiked DNA in real-time PCR. BSA did not remove inhibitors from Professor lake samples, however following treatment with EDTA, Chelex® 100 and PVP-360, DNA amplified from Professor Lake with Ct values equivalent to dd water (Table 11).

[0091] A strategy involving the addition of EDTA, Chelex® 100 and PVP-360 treatment during DNA extraction, with the addition of BSA in the real-time PCR mastermix can be applied routinely to all environmental samples when large volumes of water are analyzed. These procedures are applicable to other samples such as food and soil. The Mo Bio kit (MO BIO Laboratories Inc., Carlsberg, CA) and QIAamp® DNA stool kit (Qiagen) were also effective for inhibitor removal from environmental water samples and may be used under certain conditions. An internal control can be incorporated into the assays, based on a set of template/primers/probe distinct from all the target sequences described herein. Inclusion of an internal positive control to all real-time PCR reactions will indicate the presence of PCR inhibitors.

TABLE 11. Removal of Inhibitors from Environmental Water Samples

| Sample         | Probe           |            |
|----------------|-----------------|------------|
|                | β-giardin<br>Ct | COWP<br>Ct |
| dd Water       | 24.88±0.69      | 27.36±0.40 |
| Professor Lake |                 |            |
| Untreated      | No Ct           | No Ct      |
| Treated 1      | 24.89±0.13      | 27.61±0.19 |
| Treated 2      | 25.15±0.94      | 27.99±0.60 |
| Heart Lake     |                 |            |
| Untreated      | 23.98±0.09      | 27.09±0.35 |
| Treated        | 24.34±0.89      | 26.70±0.89 |

Real-time PCR amplification of 500pg *Giardia* ( $\beta$ -giardin) or *Cryptosporidium* (COWP) DNA in the presence of concentrated (from 2L) environmental water samples. 100  $\mu$ g/ml BSA in real-time PCR mix  
Treated samples: 0.5M EDTA, PVP-360 and Chelex® 100

## EXAMPLE 12

### Overcoming PCR Cross-contamination

[0092] To prevent cross-contamination of PCR products to yield false positives in the laboratory one can adopt the use of dUTP and uracil-N-glycosyalse (UNG). In PCR reactions dUTP becomes incorporated into the growing amplicon, rather than dTTP. At the onset of each PCR reaction a UNG treatment to cleave the uracil base from the phosphodiester DNA backbone, thus, rendering the DNA unsuitable for replication, but leaving the thymine-containing sample DNA unharmed (Longo et al., 1990).

## EXAMPLE 13

### Detection of Viable Cells

[0093] The present methodology can also be adapted to yield results for only viable cells in a sample. In particular, the presence of RNA in bacterial cells may serve as an indicator of viability, providing that the specific RNA is present only in viable cells and is degraded rapidly upon cell death. A number of studies have focused on nucleic acids associated with VBNC cells as indirect measure of cell viability (reviewed in McDougald et al., 1998). Reverse transcriptase-polymerase chain reaction assays have been developed for the detection of *L. monocytogenes* (Klein and Juneja, 1997), *V. cholerae* (Bej et al., 1996), *Mycobacterium tuberculosis* (Pai et al., 2000), *Staphylococcus aureus* and *E. coli* (McKillip et al., 1998), *E.coli* O157:H7 (Yaron and Matthews, 2002). Thus, presence of specific mRNA can serve as an indicator of metabolic activity in non culturable cells and may aid in supporting the hypothesis of VBNC.

[0094] Another approach to detecting only viable targets by PCR is DNase treatment of the bacterial cells, prior to cell lysis and DNA extraction, to rid the sample of surrounding DNA, and ensure that all DNA detected is from viable cells (Lyon, 2001).

For bacterial samples use of irreversible nucleic acid binding dyes that permeates dead cells, such as ethidium monomonoazide (EMA), could facilitate the reduction of background fluorescence signal from the DNA of dead cells (Rudi, 2002).

[0095] Viability measurements using ethidium monoazide (EMA) (Molecular Probes, Eugene, OR) treatment were carried out by the following procedure. One milliliter of 100 $\mu$ g/ml EMA in ddH<sub>2</sub>O was added to the bacteria concentrated onto filters in a vacuum filtration unit. The unit was placed in the dark for 5 min to allow the EMA to penetrate into the cells then exposed for 2.5 min to light from a 100 watt halogen light source (Oriel Inc) at a distance of 20 cm, to photo-activate the EMA. After light exposure the filters were washed with 50 ml of ddH<sub>2</sub>O, DNA was extracted and qPCR performed. A significant reduction in DNA amplification was observed when bacteria were treated at 100°C for 20 min then treated with EMA compared with EMA treatment of live cells (Table 12).

Table12: EMA treatment for Viability Determination

| Bacteria | qPCR Amplification of DNA |                |
|----------|---------------------------|----------------|
|          | EMA Treatment             |                |
|          | 0 $\mu$ g/ml              | 100 $\mu$ g/ml |
| Live     | +++                       | +++            |
| Dead*    | +++                       | -              |

\* Dead cells were obtained by treating *E. coli* for 20 min at 100°C.

[0096] A second approach involves treating the samples with EDTA to chelate out divalent cations from dead cells. This allows the collected cells to be treated with Dnase and selectively degrade dead-cell DNA. PCR amplification will occur only from viable cells.

[0097] Bacteria concentrated on the filter membranes were treated for 5 min with different concentrations of EDTA: 2mM, 0.2mM and 0.02 mM. Following treatment, the filters were washed with 50 ml of ddH<sub>2</sub>O, treated for 5 min. with 10units/ml of the Dnase (RQ1) and washed with 50 ml of water. qPCR was performed using DNA extracted from the treated cells.

## **EXAMPLE 14**

### **Detection of *Giardia* and *Cryptosporidium* in stool specimens.**

[0098] The qPCR assay was used to detect the protozoan pathogens in clinical stool specimens. *Giardia* was detected, using qPCR, in 16 clinical stool samples that were positive for *Giardia* as determined by using an immunofluorescence assay performed by the Ontario Ministry of Health parasitology Lab (Table 13). The positive specimens ranged from very low to very high levels of cysts in each patient's stool sample. The qPCR assay using the COWP primer-probe set did not detect *Cryptosporidium* in the *Giardia* positives samples. One stool specimen that was positive for *Cryptosporidium* using IFA was also positive for *Cryptosporidium* using qPCR, however, no *Giardia* were present in this sample. Thirty-six stool specimens were negative for both *Giardia* and *Cryptosporidium* as determined by both qPCR and IFA. No false positives or false negatives were observed in any of the stool specimens demonstrating the specificity and sensitivity of the qPCR assays for detecting the target pathogens.

Table 13: Real-time PCR Detection of *Giardia* and *Cryptosporidium* in Clinical Stool Specimens.

| Stool Specimens*                            | qPCR Detection (#positive/total samples) |                        |
|---|--|------------------------|
|   | <i>Giardia</i>                           | <i>Cryptosporidium</i> |
| <i>Giardia and Cryptosporidium</i> Negative | 0/36                                     | 0/36                   |
| <i>Giardia</i> Positive                     | 16/16                                    | 0/16                   |
| <i>Cryptosporidium</i> Positive             | 0/1                                      | 1/1                    |

\* The presence or absence (positive/negative) of *Giardia* and *Cryptosporidium* in the stool specimens was determined by the MOH and Mount Sinai TML parasitology laboratories, using an immunofluorescence assay (IFA).

## **EXAMPLE 15**

### **Detection of *Giardia* and *Cryptosporidium* in raw sewage.**

[0099] The qPCR assay was applied to detection of *Giardia* and *Cryptosporidium* in 1L raw sewage samples. The results were compared to detection of these pathogens using immunofluorescence assay (IFA). *Giardia* cysts were detected by qPCR at similar concentrations to IFA (Table 14). No *Cryptosporidium* oocysts were detected by either method, suggesting that the oocysts were absent or present in low numbers below our detection limit.

**Table 14: Comparison of qPCR and IFA for Detection of *G. lamblia* and *Cryptosporidium* in 1L Sewage Samples.**

| Sample <sup>a</sup> | Number of <i>G. lamblia</i> Cysts |      | Number of <i>C. parvum</i> Oocysts |     |
|---------------------|-----------------------------------|------|------------------------------------|-----|
|                     | qPCR                              | IFA  | qPCR                               | IFA |
| Negative Control    | 0                                 | -    | 0                                  | -   |
| Auteuil 1           | 5642                              | 2380 | 0                                  | 0   |
| Auteuil 2           | 10816                             | 9880 | 0                                  | 0   |
| Auteuil 3           | 13408                             | 7980 | 0                                  | 0   |
| Fabreville 1        | 2653                              | 9900 | 0                                  | 0   |
| Fabreville 2        | 5859                              | 6660 | 0                                  | 0   |
| Fabreville 3        | 3876                              | 4290 | 0                                  | 0   |

<sup>a</sup> NC, negative control in qPCR; The Auteuil and Fabreville treatment facilities, Laval, Quebec.

## **EXAMPLE 16**

### **Detection of bacteria on carcass and environmental swabs:**

[00100] Direct Detection of Bacteria on Sponges: We have tested the use of a pulsifier (Microgen Bioproducts) for its ability to dislodge bacteria from the sponge matrix and allow detection of bacteria using the qPCR assay. The pulsifier was selected over use of a stomachacher because of the efficiency of the pulsifier to detach bacteria from a matrix while causing minimal disruption of the matrix (Kang and Dougherty, 2001). Results obtained using the pulsifier for direct detection of bacteria spiked onto sponges demonstrated that greater than 70% of spiked cells were recovered when cells

were spiked onto either dry sponges or sponges hydrated with buffered peptone water (Table 15). In addition, as few as 50 *E. coli* 0157:H7 cells that were spiked onto sponges were detected.

Table 15: qPCR detection of *E. coli* 0157:H7 spiked onto sponges.

| Number of Bacteria spiked onto Sponges | % Recovery of Bacteria from Sponges* |
|--|--------------------------------------|
| Dry Sponge                             |                                      |
| 500                                    | 70                                   |
| 50                                     | 78                                   |
| Buffered Peptone Water Sponge          |                                      |
| 500                                    | 150                                  |
| 50                                     | 82                                   |

\*Percent recovery is based on the qPCR detection of bacteria spiked onto sponges then collected on a filter compared with bacteria spiked directly onto filters (positive control).

[00101] Selection of carcass swab sponges and hydration buffer: Research in the late 1980's demonstrated that certain sponge types are inhibitory to growth of bacteria in culture (Llabres and Rose, 1989). Currently, all sponges for use in bacterial detection from carcass swabs are tested to ensure they are "biocide" free, for use in detection of bacteria by cultivation. These sponges have not been tested for their suitability for use in qPCR. We conducted a study to determine whether the cellulose sponges sold by Bio International Inc. were inhibitory to qPCR. For these assays, sponges were placed in water containing 0.025% Tween 20, pulsified to dislodge material from the sponges and the homogenate collected by vacuum filtration onto filter membranes. The concentrates on the filters were extracted using Ultraprepman (ABI) extraction solution and assayed for inhibition in the qPCR assay by determining the efficiency of amplification of a known amount of purified DNA in the presence of the extracts compared to the presence of water. The dry sponges were not qPCR inhibitory, whereas, the neutralizing buffer used in environmental swabs was completely inhibitory to qPCR (Table 16). Washing the neutralizing buffer sponges overnight in ddH<sub>2</sub>O removed the qPCR inhibitory effect (Table 16).

[00102] Three buffers, commonly used to hydrate sponges for wet-swabbing of carcasses, were tested to ensure the buffers were not inhibitory to qPCR. Butterfield's

buffer, Lethen's broth and phosphate buffered peptone water were compared to hydration with ddH<sub>2</sub>O. None of the buffers were inhibitory to qPCR when added directly to the qPCR assay at a volume of 5 µl (data not shown). No difference in the Ct value was observed in the detection of DNA spiked into the PCR assay when the different buffers were compared to the ddH<sub>2</sub>O control, indicating that none of the buffers used to hydrate the sponges were inhibitory to the qPCR assay (Table 17). The qPCR assay can be used for detection of bacteria on sponges hydrated in either Lethen's, Butterfield's or buffered peptone water.

Table 16 : qPCR detection of *E. coli* 0157:H7 DNA spiked into the PCR assay in the presence of extracts from different types of sponges.

| Sponge Type                | Ct* ±SD    |
|----------------------------|------------|
| None                       | 22.11±0.18 |
| Neutralizing Buffer        | No Ct      |
| Washed Neutralizing Buffer | 22.84±0.32 |
| Dry                        | 22.55±0.49 |
| Washed Dry                 | 23.05±0.66 |

\*Ct, cycle threshold= cycle at which the detection crosses the baseline fluorescence. A Ct value indicates the presence of target DNA. No Ct indicates that no specific target DNA was present.

Table 17: Comparison of qPCR detection of DNA spiked into the PCR assay in the presence of extracts from the sponges hydrated in different buffers.

| Buffer used to Hydrate<br>Dry Sponges* | Detection of Spiked DNA<br>Ct±SD |
|--|----------------------------------|
| H <sub>2</sub> O                       | 28.55±0.49                       |
| Butterfields                           | 28.94±0.47                       |
| Lethen's                               | 28.74±0.67                       |
| Buffered Peptone Water                 | 29.08±0.66                       |

\*10ml of each buffer was used to hydrate sponges. The sponges were pulsified in 0.025% Tween water and the homogenate was concentrated through a membrane filter using vacuum filtration. Concentrated material was extracted from the filter using Ultraprepman (ABI). Each qPCR well contained 5µl of the extracted material.

Collection and concentration of bacteria from sponge swabs after growth in enrichment media:

[00103] Filter washes for media from enrichment: The following were tested to work out the optimal washes, Inhibitex Tablets from a Qiagen stool kit , PVP 40 (polyvinylpyrrolidone), EDTA (0.5 M), ETOH (25% ) and MQ Water Alone .

Effects of Washes on spent media inhibition:

[00104] A) Our results suggest that a 25% ETOH wash followed by water eliminated the inhibition with a 10 ml sample, and with a 25-ml sample. 50-ml samples collected still were inhibitory (Table 18).

Table 18: A comparison of spent and fresh media with different washes

| Sample              | Wash Treatment   | CT values(Ct± SD)        |
|---------------------|--|--------------------------|
| 1. Positive control | 10 ml water, 100 000 cells,<br>20 ml water wash                            | 22.59±0.52<br>21.88±0.32 |
| 2. Negative control | 10 ml fresh media, 100 000<br>cells, 40 ml water                           | No ct<br>No ct           |
| 3. Experimental     | 10 ml fresh media, 100 000<br>cells, 10 ml each EDTA,<br>ETOH, 20 ml water | 25.01±0.52<br>29.69±0.43 |
| 4. Negative control | 10 ml spent media+10 ml<br>PVP, EDTA, ETOH, 20 ml<br>water                 | No ct<br>No ct           |
| 5. Experimental     | 10 ml spent media, 100 000<br>cells, 10 ml PVP, EDTA,<br>ETOH, 20 ml water | No ct<br>No ct           |
| 6. Experimental     | 10 ml spent media, 100 000<br>cells, 10 ml each EDTA,<br>ETOH, 20 ml water | 22.57±0.21<br>24.69±0.21 |
| 7. Experimental     | 10 ml spent media, 100 000<br>cells, 10 ml ETOH, 20 ml<br>water            | 20.09±0.34<br>21.19±0.43 |
| 8. Experimental     | 10 ml spent media, 100 000<br>cells, 10 ml EDTA, 20 ml<br>water            | No ct<br>No ct           |
| 9. Experimental     | 10 ml spent media, 100 000<br>cells, 50 ml water                           | No ct<br>No ct           |

[00105] B) Our results suggest that 35 ml media (TSB) with 500cells on sponge with of 25 ml ETOH and 100 ml of water washes gave a good Ct value (Table 19)

Table 19: Time points for enrichment of media with sponges

| Sample              | Wash Treatment   | CT value<br>4hr | CT value<br>5hr | CT value<br>6hr |
|---------------------|--|-----------------|-----------------|-----------------|
| 1. Positive control | 25 ml water, 100, 000 cells, 50 ml water wash              | -               | 21.67±0.52      | 22.17±0.43      |
| 2. Media control    | 35 ml media, 25 ml ETOH and 100ml of water                 | No ct           | No ct           | No ct           |
| 3. Experimental     | 35 ml media +500cells on sponge , 25 ml ETOH, 100 ml water | 23.54±0.52      | 22.72±0.43      | 21.76±0.53      |

[00106] Protocol for measuring from samples:

1. Sponge swabs will be put into 125 ml nutrient broth or TSB media, and incubated at 37°C
2. At some time point 2-5 hours after incubation, the media will be divided into three aliquots, 25 ml for culturing, and up to 50 ml for qPCR
3. The procedure for washing and collection is described above.

[00107] Although the foregoing invention has been described in some detail by way of illustration and examples for the purposes of clarity , one skilled in the art will appreciate that certain changes and modifications may be practiced within the scope of the invention as defined by the appended claims.

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[00108] Articles referenced herein, and incorporated herein by reference, are listed below:

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[00109] Various embodiments of the present invention having been thus described in detail by way of example, it will be apparent to those skilled in the art that variations and modifications may be made without departing from the invention. The invention includes all such variations and modifications as fall within the scope of the appended claims.